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# Densely Methylated DNA Islands in Mammalian Chromosomal Replication Origins†

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**Densely methylated DNA sequence islands, designated DMIs, have been observed in two Chinese hamster cell chromosomal replication origins by using a PCR-based chemical method of detection. One of the origins, $ori_{S14}$, is located within or adjacent to the coding sequence for ribosomal protein S14 on chromosome 2q, and the other, *ori*-β, is ~17 kbp downstream of the *dhfr* (dihydrofolic acid reductase) locus on chromosome 2p. The DMI in $ori_{S14}$ is 127 bp long, and the DMI in *ori*-β is 516 bp long. Both DMIs are bilaterally methylated (i.e., all dCs are modified to 5-methyl dC) only in cells that are replicating their DNA. When cell growth and DNA replication are arrested, methylation of CpA, CpT, and CpC dinucleotides is lost and the sequence islands display only a subset of their originally methylated CpG dinucleotides. Several possible roles for DMI-mediated regulation of mammalian chromosomal origins are considered.**

To investigate the relationship between DNA cytosine methylation and site-specific point mutations in mammalian genes, we analyzed the positions of cytosine methylation in genomic DNA encoding the cloned Chinese hamster ovary (CHO) cell gene for ribosomal protein S14 (*RPS14*) and compared the methylation sites detected with the position of a recurrent transition mutation affecting the gene's fifth exon (39). During the course of that study, we noted an unusual methylation pattern within a short stretch of the DNA sequence at the 3′ end of the gene. Although cytosine methylation at the 5′ end and middle of CHO *RPS14* occurs exclusively at CpG dinucleotides, in exponentially growing cells actively engaged in DNA replication, we found a unique chromosome segment at the 3′ end of the gene in which every dC residue was methylated. Within this densely methylated island (DMI), both DNA strands were fully methylated, and 5-methyl cytosines were detected in CpA, CpT, and CpC as well as CpG dinucleotides.

In the accompanying paper, we report that the 3′ end of the CHO *RPS14* locus also encodes an early-S-phase origin of bidirectional chromosomal DNA replication (OBR) designated $ori_{S14}$ (40). Because DNA sequence motifs containing methylated adenine residues are essential components of prokaryotic chromosomal OBRs (2, 22, 29) and methylation deficiency mutations in *Neurospora crassa* lead to postreplicative chromosomal anomalies (12), we have investigated the possibility that DMIs are important for the regulation of programmed DNA replication in mammalian cells.

Now we describe DMIs in two mammalian chromosomal OBRs: $ori_{S14}$ and *ori*-β downstream of the Chinese hamster *dhfr* (dihydrofolic acid reductase) locus (1, 6, 18, 20, 42, 43). Both DMIs contain unique termini and are fully methylated in both DNA strands. The DMI in $ori_{S14}$ ($DMI_{S14}$) is 127 bp in length, and the DMI in *ori*-β ($DMI_{dhfr}$) is 516 bp long. Methylation of CpA, CpT, and CpC dinucleotides in both DMIs is lost rapidly when mitotic growth and DNA replication are arrested. Only a few methylated CpG dinucleotides are found in the DMIs of cells deprived of serum growth factors or required amino acids. Conversely, when growth and DNA replication are restored by refeeding the cells with optimal culture medium or by viral transformation, CpA, CpT, and CpC dinucleotides in the DMIs are remethylated rapidly.

Three possible functions for the DMIs in mammalian chromosomal OBRs are considered: (i) association of chromosomal OBRs with the nuclear matrix or inner nuclear envelope, (ii) the licensing or activation of particular replication origins during specific developmental programs, and (iii) the use of DMIs as molecular signposts to mark previously replicated chromosomal origins.

## MATERIALS AND METHODS

**Materials.** Tissue culture medium (Dulbecco's modified Eagle's medium) and fetal bovine serum were products of GIBCO BRL Life Technologies, Inc. *Escherichia coli* DNA polymerase I (Klenow fragment), *Taq* polymerase, M13 forward and reverse sequencing primers, T7 and SP6 promoter oligonucleotides, and restriction endonucleases were purchased from Promega Corp. 5-Methyl dCTP was from Pharmacia LKB Biotechnology. [α-$^{32}$P]dCTP (3,000 Ci/mmol) was obtained from DuPont NEN Research Products. Commercial sources for all other enzymes and reagents used have been described before (39, 40).

**Tissue culture.** A clone of CHO-K1 cells (ATCC CCL61) that carries mutational markers for proline and glycine auxotrophy (17) was used in these studies. The cells were maintained in monolayer culture at 37°C in antibiotic-free Dulbecco's modified Eagle's medium plus proline and glycine ($10^{-4}$ M each) supplemented with 10% (vol/vol) fetal bovine serum.

**DNA sequences.** A full-length genomic clone of Chinese hamster *RPS14* carried in plasmid pBSKM13+ (Stratagene), pGS14-45, has been described previously (33). This clone's DNA derives from a portion of chromosome 2q rendered hemizygous in CHO-K1 cells by a large interstitial deletion (46). Its complete nucleotide sequence (5,300 bp) is deposited in GenBank (accession number M35008) under the locus name CRURPS14A. A plasmid clone, pX24, encoding ~4.5 kbp of the Chinese hamster *ori*-β region (5) was generously provided by M. DePamphilis and D. Gilbert (Roche Institute of Molecular Biology). The genomic DNA sequence (6,157 bp) of CHO

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TABLE 1. Oligonucleotide primers used in this study

| Primer | Methyl design[a] | Location[b] | Sequence[b] |
|---|---|---|---|
| *RPS14* | | | |
| Sense strand | | | |
| Pa | + | 4695–4716 | 5′-ATGGGCAATGACATTTTTTCTT-3′ |
| P1 | − | 4721–4742 | 5′-TTGAATTCTAGAGGATGTTATTTTTATTTT-3′ |
| P2 | − | 4879–4860 | 5′-ATGGATCCAAATCCTACCTCAAAACAAA-3′ |
| Antisense strand | | | |
| P3 | − | 4732–4753 | 5′-AAGAATTCACCCCCATCCCCTCTAACAACA-3′ |
| Pb | + | 4919–4901 | 5′-CTGAGTTGGAGGCCAGACT-3′ |
| P4 | − | 4929–4908 | 5′-AGGGATCCGTATGTGGATTTGAGTTGGAGG-3′ |
| *dhfr ori-β*[c] | | | |
| Sense strand | | | |
| P5 | − | 2631–2660 | 5′-AAGAATTCGTTTTAAATAAGATTTATTTG-3′ |
| Pc | + | 2944–2970 | 5′-CTTTTTCAACTGGGAAATCATTCAAGG-3′ |
| P6 | − | 3492–3461 | 5′-ACGGATCCCTAATTTAAACTAATAATAATA-3′ |
| P7 | + | 2891–2913 | 5′-TTGAATTCATTGGGAAATCATTCAAGGATG-3′ |
| P8 | + | 3423–3394 | 5′-AAGGATCCTTACTAAATCTCATCAGAAATA-3′ |
| Antisense strand | | | |
| P9 | +/− | 2570–2598 | 5′-TAGAATTCAGGGCAACTAAAACACATAAT-3′ |
| P10 | − | 2889–2860 | 5′-AGGGATCCTGTTTTTATGTTGATATTTGT-3′ |
| P11 | + | 2798–2817 | 5′-AAAGTTCAGCCTTAACCTTT-3′ |
| P12 | + | 3400–3381 | 5′-AGAAATAGCAAGCTGGGGTA-3′ |
| Pd | + | 3220–3196 | 5′-CAATGGATTAAACCTCTGAACTGT-3′ |
| P13 | − | 3331–3359 | 5′-TAGAATTCAAACCATTTTCATTCAAACCA-3′ |
| P14 | − | 3659–3631 | 5′-TTGGATCCAAGGTAGGTAGATTATTGTGA-3′ |
| *tk* | | | |
| Sense strand | | | |
| P15 | − | 1–29 | 5′-TGGAATTCTTTATAGGTGGAGGTGATTGG-3′ |
| P16 | − | 390–361 | 5′-AGGGATCCACTAACTCTAATCTAAACTAAA-3′ |
| Antisense strand | | | |
| P17 | − | 1–29 | 5′-TAGAATTCTCCACAAATAAAAATAATTAA-3′ |
| P18 | − | 390–361 | 5′-AGGGATCCGTTGGTTTTAGTTTGGGTTAAA-3′ |
| *mtII* | | | |
| Sense strand | | | |
| P19 | − | 750–779 | 5′-TGGAATTCTTTTTTTGTTTATGGTTAATTT-3′ |
| P20 | − | 1126–1095 | 5′-CTGGATCCCCAAACCACAAACTAATATTTA-3′ |
| Antisense strand | | | |
| P21 | − | 750–779 | 5′-TAGAATTCTTTTTCCACTCACAATCAACTC-3′ |
| P22 | − | 1126–1095 | 5′-TTGGATCCTTAAGTTATAAATTAGTATTTG-3′ |

[a] Oligonucleotide sequences were designed on the assumption that genomic DNAs were either fully methylated (+) or fully demethylated (−). In the latter case, bisulfite treatment quantitatively converts dC residues to dT.

[b] Taken from the GenBank entry (see text). *Bam*HI (G ↓ GATCC) and *Eco*RI (G ↓ AATTC) cleavage sites (underlined) were introduced into the 5′ ends of most primers to facilitate cloning of their amplification products.

[c] Sense strand refers to the DNA strand that encodes the upstream DHFR polypeptide, and antisense strand refers to the complementary DNA strand.

*ori*-β on chromosome 2p (8) is listed in GenBank under the locus name CGDHFRORI (accession number X52034). DNA sequences of Chinese hamster thymidine kinase (*tk*) exon VII and metallothionein II (*mtII*) exon III also were obtained from the GenBank database (accession numbers L00369 and X55065, respectively). Nucleotide residues in all of these sequences are numbered according to the indices used in their GenBank entries.

Primer oligonucleotides for PCR amplification were synthesized by the Kansas State University Biotechnology Core Facility (Table 1). Oligonucleotides used to amplify sodium bisulfite-treated DNAs (see below) were designed on the assumption either that none of the dC residues within the priming sequences were methylated and therefore were converted to dTs through bisulfite modification or that all dCs were methylated and therefore remained dCs. In designing one primer, P9 (Table 1), we found it necessary to use an intermediate strategy in which only some of the dCs were converted to dTs to obtain site-specific PCR amplification. Most of the primer oligonucleotides contained either a *Bam*HI (G ↓ GATCC) or an *Eco*RI (G ↓ AATTC) site near their 5′ ends to facilitate the subsequent cloning of amplification products into pUC-13. Two of the primers, P11 and P12, lacked restriction endonuclease cleavage sites, and their amplification products were cloned into pGEM-T (Promega).

**Genomic DNA preparations and analysis of 5-methyl dC.** High-molecular-weight (>50 kbp) CHO cell genomic DNAs were purified from cells grown in monolayer culture as described previously (27, 28). Chromosomal DNAs were analyzed for 5-methyl dC by using a PCR-based chemical procedure (13, 39). As described originally by Frommer et al. (13), high-molecular-weight DNA can be treated with sodium bisulfite under conditions which quantitatively convert dCs, but not 5-methyl dCs, to dUs (36, 37, 44). Chemical modification permits a PCR-based assay in which 5-methyl dCs yield positive signals (dC bands) in DNA sequence ladders in which unmethylated dC residues are altered to dTs. Since 5-methylcytosine is the only modified pyrimidine base observed in eukaryotic DNAs, this strategy provides a sensitive, sequence-independent assay for detection of 5-methylcytosine in complex DNAs (13, 39). Accordingly, genomic DNAs were fragmented by partial digestion with *Hin*dIII, denatured in alkali,

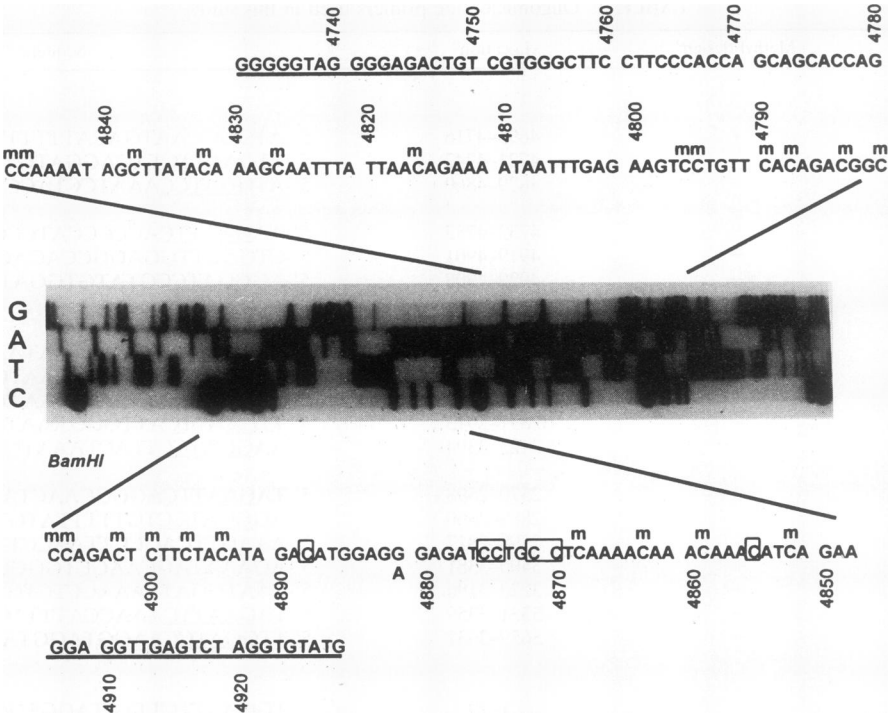


FIG. 1. CHO *RPS14* contains a 127-bp densely methylated DNA motif. The DNA sequence ladder illustrated derives from CHO cell genomic DNA spanning *RPS14* exon V (top) through the proximal downstream flanking sequence (bottom). Primers P3 and P4 (listed in Table 1 and underlined in the figure) were used to amplify the bisulfite-modified antisense strand of *RPS14* (residues 4732 to 4929), and a pUC13 clone of the amplified sequence was sequenced through primer P4's *Bam*HI site by using the M13 reverse sequencing primer. Note that 22 of 28 dCs between positions 4781 and 4907 are contained within the sequence ladder shown and are represented as $\overset{m}{C}$, indicating that they are methylated in the chromosomal locus. The six dCs which are not methylated in the cloned molecule sequenced for this figure are highlighted by rectangles. The DNA molecule whose sequence is shown contains a G at residue 4881, in contrast to the sequence listed in GenBank, in which an A was detected at this position. Because other molecules cloned from the same PCR amplification used to generate this figure were approximately equally divided between DNAs with A's and G's at this position, it appears that this observed polymorphism reflects a PCR amplification artifact.

recovered by alcohol precipitation, and treated with 1 M sodium bisulfite in 0.5 mM hydroquinone (pH 5.0) for 48 h at 50°C in a programmable thermocycler (39). To maintain the DNAs in a single-stranded form, which enhances their sensitivity to bisulfite modification, the reaction temperature was raised to 95°C for 5 min every 3 h. Modified DNAs were purified by dialysis and treated for 10 min with 0.3 M NaOH at 22°C to remove the bisulfite adducts and complete the conversion of dCs to dUs. Finally, the DNAs were recovered by ethanol precipitation and stored in 0.01 M Tris (pH 7.2)–0.001 M EDTA.

Individual chromosome loci in bisulfite-modified genomic DNAs were amplified by PCR using gene-specific, synthetic oligonucleotide primers. PCR primers were designed for most genomic DNA sequences on the assumption that dCs had been quantitatively converted to dUs (dTs) by bisulfite treatment. However, for genomic sequences within densely methylated DNA motifs, oligonucleotides were synthesized without this assumption. Because the two strands of bisulfite-treated duplex DNA are rendered noncomplementary by bisulfite treatment, genomic DNA strands must be amplified individually by using two different pairs of strand-specific oligonucleotide primers (Table 1).

**PCR amplification and DNA sequence analysis.** PCR amplification mixtures (50 μl) contained 10 ng (30 pmol) of bisulfite-treated or control genomic DNA, 120 ng (360 pmol) of each of two locus-specific synthetic oligonucleotide primers, *Taq* polymerase (2.5 U), and the buffer components described previously (39). The template DNAs were amplified through 30 iterations of a reaction cycle consisting of 1 min of denaturation at 94°C, 1 min of annealing at 50°C, and 2 min of elongation at 72°C. Fully methylated and fully demethylated control DNAs were prepared from CHO *RPS14* exon V and proximal downstream flanking DNA by PCR amplification in reactions that contained either 5-methyl dCTP or dCTP, respectively (39).

Amplified DNA products were cloned into the plasmid vector pUC-13 or pGEM-T and recovered in *E. coli* TB1 (GIBCO-BRL Life Technologies). Individual clones of the amplified DNA fragments were sequenced by using the dideoxynucleotide chain termination method (34), *E. coli* DNA polymerase I (Klenow fragment), and the M13 sequencing and reverse sequencing primers (for pUC-13) or T7 and SP6 oligonucleotide primers (for pGEM-T) (21, 30, 31, 33).

## RESULTS

**The 3′ end of CHO *RPS14* contains a densely methylated DNA island.** In a recent study, we analyzed the locations of 5-methyl dC within exon V of the Chinese hamster *RPS14* locus (39) to investigate the molecular mechanism responsible for a high-frequency, recurrent transition mutation in the CHO S14-coding sequence (10). During the course of our studies, we noted an unusual cytosine methylation pattern within a 127-bp chromosomal sequence spanning the 3′ half of *RPS14* exon V and proximal downstream flanking DNA.

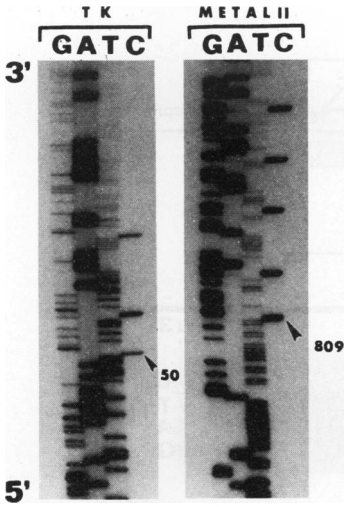


FIG. 2. The CHO *tk* and *mtII* loci do not contain densely methylated DNA motifs near their 3′ termini. CHO genomic DNA was treated with sodium bisulfite, and coding-strand fragments of the *tk* (TK) and *mtII* (METALII) loci were PCR amplified and cloned into pUC-13. Genomic DNA encoding *tk* exon VII and *mtII* exon III were amplified by using primers P15 plus P16 and P19 plus P20, respectively (Table 1). Reference positions, numbered according to the GenBank entries, are marked by arrows near the 5′ ends (bottom) of the two sequence ladders. Note that the only dC residues which remain in the DNAs are those within CpG dinucleotides, i.e., 5-methylcytidine residues.

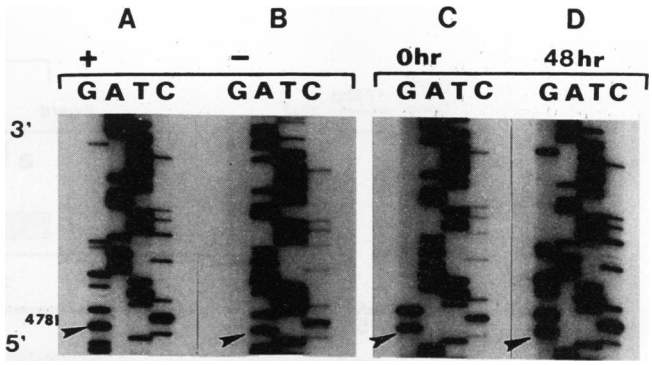


FIG. 3. The DMI in $ori_{S14}$ is rapidly remethylated when growth-arrested CHO cells reinitiate DNA replication. (A) Fully methylated control DNA derived from the 3′ end of *RPS14*; (B) demethylated control DNA from the same region of *RPS14*. Nuclear DNA was purified from one sample of cells following 2 days of maintenance in medium containing 0.5% fetal bovine serum (C) and from another sample of cells after 48 h of growth stimulation in medium supplemented with 10% serum (D). The DNA samples were treated with sodium bisulfite and PCR amplified by using primers P3 and P4 (Table 1). DNA sequence ladders shown in all four panels (positions 4777 to 4824) were determined through the *Eco*RI site in primer P3 (Table 1) by using the M13 forward sequencing primer. Accordingly, the sequences illustrated are complementary to antisense chromosomal DNA, and conversion of chromosomal dCs to dTs is evidenced by complementary dG→dA replacements in the figure. The location of the dG or dA at position 4781 (the 5′ end of the DMI) is marked by arrowheads.

As illustrated by the cloned molecule whose sequence is shown in Fig. 1, most dCs (22 of 28) in the antisense strand of CHO genomic *RPS14* DNA between residues 4781 and 4907 are resistant to sodium bisulfite in DNA purified from exponentially growing cells. For this reason, they appear to be methylated and therefore are represented as $\overset{m}{C}$ in Fig. 1. In other cloned molecules of amplified bisulfite-treated DNA whose sequences were analyzed, the six dCs not methylated in the molecule illustrated in Fig. 1 (highlighted by boxes) were present as 5-methyl dC, and a few other dCs (three to six) were unmethylated. Cytosines immediately outside of this sequence segment are not methylated in any of the cloned DNAs whose sequences were analyzed and therefore were converted to thymines by sodium bisulfite treatment and PCR amplification. We also PCR amplified the bisulfite-modified sense strand of *RPS14* (residues 4721 to 4879) by using primers P1 and P2 (Table 1) and obtained identical results (data not shown). This indicated that both chromosomal DNA strands encoding *RPS14* residues 4781 to 4907 are heavily methylated, as most, if not all, dC residues in this chromosome segment are replaced by 5-methylcytidine. Elsewhere we have reported that 5-methylcytidine residues in 5′ end and middle segments of the CHO chromosomal *RPS14* locus occur exclusively within CpG dinucleotides (39). In contrast, within the CHO cell chromosomal DNA sequence segment described above, all dCs are methylated. Data to support this conclusion derive from three separate preparations of bisulfite-treated genomic DNA, seven independent PCR amplifications, and the sequencing of 50 plasmid clones carrying DNA inserts amplified from both genomic strands of $ori_{S14}$. Additionally, the *RPS14* locus in another Chinese hamster cell line, V79 (14), also displays exactly the same densely methylated DNA sequence motif (data not shown). Hereafter, we refer to this unusual methylation pattern as a DMI. The double-stranded DMI at the 3′ end of the CHO S14 gene contains 48 5-methylcytidine residues, of which 18 (37.5%) are within CpA dinucleotides, 7 (14.6%) are within CpC dinucleotides, 4 (8.3%) are within CpG dinucleotides, and 19 (39.6%) are within CpT dinucleotides.

**Human *RPS14* exon V and two other CHO genes do not contain DMIs.** To determine whether the DMI in *RPS14* exon V is conserved in another mammalian species, we used a set of primers similar to those listed in Table 1 to analyze the methylation status of exon V and proximal downstream DNA in the *RPS14* locus carried by four established human cell lines (HeLa S3, HL60, CCRF-CEM, and HT-1080) as well as primary human placental cells. The structure and complete nucleotide sequence of the human *RPS14* locus have been described previously (30) and are listed in GenBank under accession number M13934. In all of the human DNA samples, only five 5-methylcytidine residues were observed, and all five were contained in CpG dinucleotides (data not shown). We concluded, therefore, that a DMI at the 3′ end of the *RPS14* locus is not essential for a functional mammalian S14 gene, as it has not been conserved in human *RPS14*. Similarly, cloned human and Chinese hamster *RPS14* transgenes are actively replicated and transcribed when stably introduced into mammalian tissue culture cells despite the fact that they do not retain densely methylated islands after propagation in bacteria (32). Interestingly, the human S14 locus encodes a different, 257-bp DMI at the 5′ end of its first intron, and this DMI maps within or near yet another apparent chromosomal replication origin (40a).

Next we examined whether DMIs can be detected at the 3′ ends of other transcriptionally active CHO chromosomal loci. We chose two genes whose 3′ end sequences are known: *tk* and *mtII*. Figure 2 illustrates representative sequence ladders obtained from the protein-coding (i.e., sense) strands of both

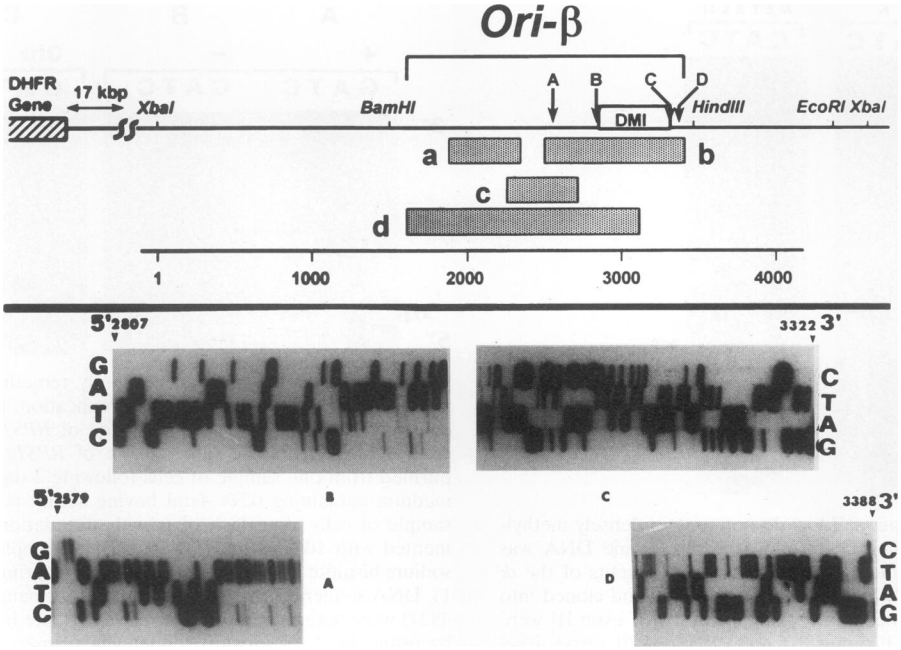


FIG. 4. CHO *ori*-β contains a 516-bp DMI. In panels B and C, a portion of the CHO cell chromosomal region encoding *ori*-β (residues 2798 to 3400) was PCR amplified from bisulfite-treated genomic DNA, using primers P11 and P12 (Table 1). In addition, a 290-bp segment of flanking upstream *ori*-β DNA (residues 2570 to 2889) was amplified with primers P9 and P10 (A), and a 328-bp fragment of downstream *ori*-β DNA (residues 3331 to 3659) was amplified with primers P13 and P14 (D). At the top is a restriction map of the genomic *Xba*I DNA fragment cloned in plasmid pX24 (5). The hatched rectangle at the left represents the 3′ end of the *dhfr* locus. The nucleotide numbering system illustrated below the map was taken from Caddle et al. (8). Sites within the map labeled A to D refer to the 5′-most positions read in the DNA sequence ladders illustrated in panels A to D (i.e., positions 2579, 2807, 3322, and 3388, respectively). A 516-bp DMI located between positions 2807 and 3322 is represented by an open rectangle above the restriction map. Map locations of the *ori*-β initiation zone, as determined by several biochemical strategies, are represented by stippled rectangles below the map (a, from Anachkova and Hamlin [1]; b, from GenBank entry X52034 as prepared by N. Heintz [University of Vermont]; c, from Burhans et al. [6]; d, from Vassilev et al. [42]). Sequence ladders illustrated in panels A and B were generated by using the M13 forward sequencing primer and therefore show the complement to the upstream *dhfr* antisense DNA sequence. For this reason, 5-methylcytidine residues in genomic DNA are represented as complementary dG bands in these sequence ladders. The three G bands seen in panel A derive from primer P9 (Table 1). The DNA ladders in panels C and D were generated by using the M13 reverse sequencing primer and can be read directly.

genes following modification of the DNA with sodium bisulfite. As shown, a portion of CHO *tk* exon VII contains three 5-methylcytidine residues (positions 50, 59, and 83), all of which map to the CpG dinucleotides. Similarly, the part of *mtII* exon III illustrated contains five 5-methylcytidine residues (positions 809, 821, 840, 857, and 876) also located exclusively at CpG dinucleotides. In neither case were 5-methylcytidine residues detected within CpA, CpC, or CpT sequences, and in both gene segments, the great majority of dCs analyzed (112 dCs in the *tk* locus and 82 dCs in the *mtII* locus) were not methylated and therefore represented as dTs in PCR amplification products. From the data shown in Fig. 2, as well as similar information derived from the two genes' chromosomal antisense DNA strands (not shown), we concluded that DMIs are not located within all transcriptionally active loci in CHO-K1 cells.

**Methylation of the *RPS14* DMI is dynamically regulated with DNA replication.** Inasmuch as the DMI described above maps within the chromosomal OBR designated $ori_{S14}$ (40), and because CpA, CpC, and CpT methylations in the same chromosomal region are not present in replication-arrested $G_0$-phase CHO cells (39), it seemed likely that methylation within the DMI is dynamically regulated with the cells' program of DNA replication.

The data in Fig. 3 demonstrate that the *RPS14* DMI is rapidly demethylated when cellular growth and DNA replication are arrested by serum deprivation and quickly remethylated when the arrest is reversed. DNA sequence ladders shown in Fig. 3 were prepared from the complement to antisense DNA clones of bisulfite-treated DNA. Therefore, chemical modification of dCs to dTs in the original chromosomes are evidenced by dG→dA changes in the sequences illustrated. Following 48 h of serum deprivation, the segment of the DMI sequence shown in Fig. 3C contains only two dG residues, both located in CpG dinucleotides. This indicates that CpA, CpC, and CpT dinucleotides had been demethylated during 2 days of maintenance in medium with 0.5% fetal bovine serum. However, 48 h after the cells were induced to resume DNA replication by refeeding with growth medium containing 10% fetal bovine serum (Fig. 3D), all dCs within the DMI were remethylated and therefore are represented by dG bands in Fig. 3.

The experiment whose results are shown in Fig. 3 was repeated by amplifying both strands of chromosomal DNA with primers P1 to P4 (Table 1) and by sequence determinations on ≈60 DNA clones, using both the M13 forward and reverse sequencing primers. Also, DNA replication was reversibly arrested by depriving cells of a required amino acid (proline) and restored in medium containing 0.5% serum by transformation with the papovavirus simian virus 40 (data not shown). In all cases, the same results were obtained. Therefore, we conclude that methylation of CpA, CpC, and CpT

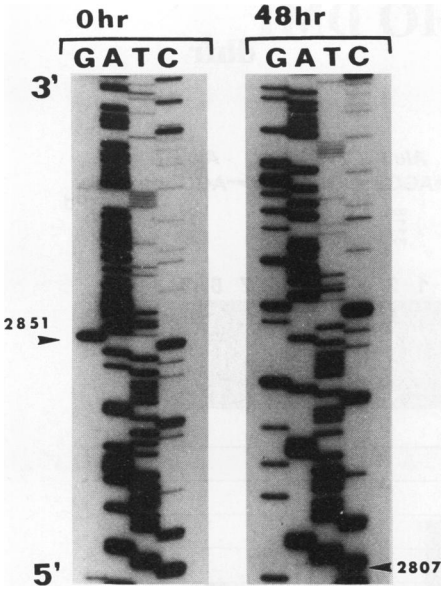


FIG. 5. $DMI_{dhfr}$ methylation is dynamically regulated with the cell's DNA replication program. Genomic DNAs were isolated from CHO cells following 2 days of maintenance in medium containing 0.5% fetal bovine serum (0hr) and after 48 h of restimulation in complete growth medium with 10% serum (48hr). After treatment with sodium bisulfite, antisense DNA segments encoding $DMI_{dhfr}$ were PCR amplified with primers P11 and P12 (Table 1). Sequences shown are complementary to the genomic antisense DNA strand and were generated by using the M13 forward sequencing primer. Accordingly, 5-methylcytidine residues are marked by dG bands in the ladders illustrated. The single G band observed in the 0-h sample is at position 2851 within a CpG dinucleotide. The 5′ end of the DMI at position 2807 is marked by an arrowhead to the right of the 48-h sample.

dinucleotides in the *RPS14* DMI is reversible in cultured cells, that the DMI is fully methylated in cells which are actively replicating chromosomal DNA, and that the DMI is not methylated in cells when growth and DNA replication are arrested.

**The *dhfr* replication origin, *ori*-β, also contains a long DMI.** From the data described above, it seemed likely that the densely methylated DNA motif detected in $ori_{S14}$ plays an important role in either the function or regulation of a mammalian chromosomal OBR. If this were true, then other mammalian OBRs also should include DMIs. The most thoroughly characterized mammalian OBR is the Chinese hamster *ori*-β site ~17 kbp downstream of the *dhfr* locus on chromosome 2p (1, 6, 18, 20, 42, 43). *ori*-β has been mapped biochemically by analysis of imbalanced DNA replication (7, 15), nascent DNA strand lengths (42), and Okazaki fragment distribution (6) to a short segment of cloned DNA whose complete base sequence was determined (8). On the basis of this sequence, we designed a comprehensive set of PCR amplification primers to analyze the distribution of genomic 5-methylcytosine within *ori*-β (primers P5 to P14; Table 1).

The diagram at the top of Fig. 4 illustrates that *ori*-β is located within a 4.5-kbp *Xba*I DNA fragment which has been cloned in a bacterial plasmid designated pX24 (5). As described above, several biochemical methods were used to locate the OBR's initiation zone within a 1.7-kbp segment of this fragment (residues 1700 to 3400; Fig. 4). We detected a 516-bp DMI in *ori*-β between positions 2807 and 3322 (sites B and C; Fig. 4). Because the DMI is too long to illustrate convincingly with a single DNA sequence ladder, the *dhfr*-proximal (Fig. 4B) and *dhfr*-distal (Fig. 4C) ends of the DMI as well as neighboring upstream (Fig. 4A) and downstream (Fig. 4D) chromosome segments are shown as individual sequence ladders. As indicated by the presence of dG bands in Fig. 4B and dC bands in Fig. 4C, the chromosomal DNA sequence between positions 2820 and 3300 is densely methylated. Outside of this region, most of the dCs are unmethylated (Fig. 4A and D); the few that are methylated reside within CpG dinucleotides. We have designated this densely methylated sequence island as $DMI_{dhfr}$ to distinguish it from the DMI within *RPS14* ($DMI_{S14}$). As was observed in $DMI_{S14}$, the DMI in *ori*-β includes 5-methylcytosine within CpA, CpC, and CpT as well as CpG dinucleotides in both chromosomal DNA strands. Methylation of $DMI_{dhfr}$ also is dynamically regulated with the cells' replication program (Fig. 5).

**The DMIs within $ori_{S14}$ and *ori*-β are not cleaved by a methylation-sensitive restriction endonuclease.** To confirm the presence of dense cytosine methylation within $ori_{S14}$ and *ori*-β and to determine the fractions of chromosomal DNAs that are densely methylated at these sites, we performed the experiments whose results are summarized in Fig. 6. CHO cell genomic DNA was digested either with *Alu*I, a restriction endonuclease that does not cleave methylated DNA sequences (35), or with *Mbo*I or *Mbo*II, both of which are insensitive to the target DNA's methylation status. In preliminary experiments, we observed that *Alu*I cleaves these two DMI sequences when demethylated by PCR amplification or by propagation as recombinant DNAs within bacterial hosts (data not shown). Digested DNAs were used as templates to PCR amplify $DMI_{S14}$ (Fig. 6A) and $DMI_{dhfr}$ (Fig. 6B). Undigested DNA templates also were amplified to verify that the amount of amplification product obtained was proportional to the amount of intact DNA template introduced into each PCR. We predicted that *Mbo*I and *Mbo*II would cleave the DMIs even when methylated and therefore would destroy their PCR template activities. In contrast, *Alu*I would not cleave methylated DMI target sequences. As illustrated in Fig. 6, these expectations were satisfied. Although *Mbo*I (Fig. 6A, lane 3) and *Mbo*II (Fig. 6B, lane 3) cleaved the genomic DNA template and abolished its ability to direct PCR amplification at the two DMI sites examined, *Alu*I only slightly reduced the chromosomal DNA's template activities (see below).

Inasmuch as the PCR conditions used appeared to be at least semiquantitative (Fig. 6A and B, lanes 4 to 9), we were able to use PCR amplification to estimate the fractions of chromosomal DNA resistant to *Alu*I digestion within the two DMIs. Accordingly, both gels shown in Fig. 6 were analyzed by soft laser densitometry, and the results are illustrated in the bar graphs below the gels. Comparison of the amplification products derived from *Alu*I-digested template (lanes 2) with the products obtained from the same amount of control DNA template (lanes 5) indicated that 86% of the DNAs encoding $DMI_{S14}$ and 92% of the DNAs specifying $DMI_{dhfr}$ resisted *Alu*I digestion and therefore appeared to be heavily methylated. The remaining 8 and 14% of the chromosomal DNAs (for $DMI_{dhfr}$ and $DMI_{S14}$, respectively) are likely to carry incompletely methylated DMIs such as discussed with respect to the cloned molecule illustrated in Fig. 1 (see above).

## DISCUSSION

We have described unusual, densely methylated DNA sequence islands, referred to as DMIs, within two mammalian chromosomal replication origins. Most, if not all, dC residues on both DNA strands of the DMIs are methylated within CpA,

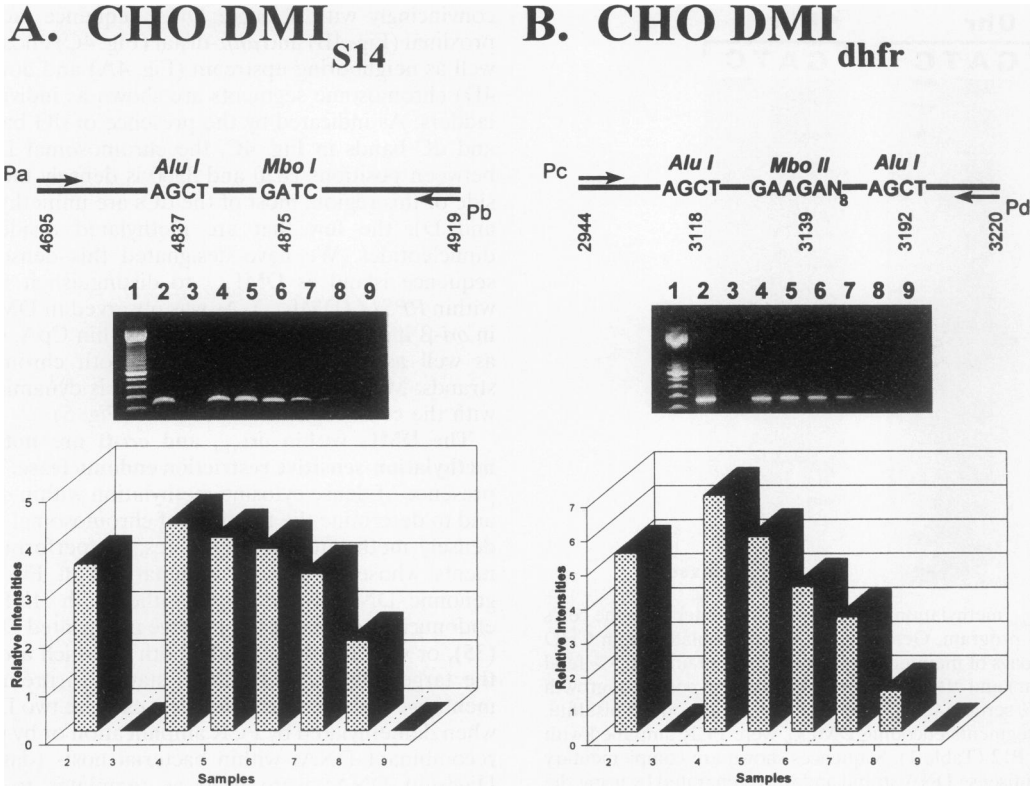


FIG. 6. CHO $DMI_{S14}$ and $DMI_{dhfr}$ are resistant to digestion with a methylation-sensitive restriction endonuclease. CHO cell genomic DNA was digested with *Alu*I, *Mbo*I, or *Mbo*II prior to 30 cycles of PCR amplification. PCRs were carried out as described in Materials and Methods. The locations and structures of the oligonucleotide primers used (Pa, Pb, Pc, and Pd) are listed in Table 1. As indicated by the diagram in panel A (not drawn to scale), oligonucleotides Pa and Pb prime amplification of a 225-bp *RPS14* DNA fragment that includes $DMI_{S14}$ and contains *Alu*I and *Mbo*I cleavage sites at residues 4837 and 4874, respectively. The diagram shown in panel B illustrates that oligonucleotides Pc and Pd prime amplification of a 277-bp DNA segment of $DMI_{dhfr}$. This DNA fragment includes two *Alu*I cleavage sites (positions 3118 and 3192) as well as an *Mbo*II site (position 3139). *Alu*I does not cleave methylated DNA targets (35), whereas *Mbo*I and *Mbo*II do. PCR amplification products were resolved by electrophoresis through 1.5% agarose gels, stained with ethidium bromide, and photographed, and the photographic negatives were analyzed by soft laser densitometry to obtain quantitative data summarized in the two bar graphs. In panels A and B, lanes 1 were loaded with molecular size markers (100-bp ladders), lanes 2 contained the products amplified from *Alu*I-digested genomic DNA (175 ng), and lanes 4 to 9 were loaded with the PCR products amplified from 225, 175, 125, 75, 25 and 0 ng of intact genomic DNA template, respectively. Lanes 3 contained the PCR products amplified from *Mbo*I-digested (A) and *Mbo*II-digested (B) genomic DNA template (175 ng). Comparison of the amplification products detected in lanes 2 and 5 indicate that 86 and 92% of the chromosomal DNAs encoding $DMI_{S14}$ and $DMI_{dhfr}$ resisted *Alu*I digestion and therefore appeared to carry methylated *Alu*I cleavage sites.

CpC, and CpT as well as CpG dinucleotides. Two lines of evidence suggest that DMIs are important components of chromosomal replication origins in higher animals. First, DMIs were observed in the chromosomal sequences encoding two cloned Chinese hamster replication origins ($ori_{S14}$, located within the *RPS14* locus, and *ori*-β, situated near the *dhfr* gene) as well as in a human chromosomal replication origin located in the first intron of the human *RPS14* locus (40a). Several other cloned genetic loci, including CHO-K1 *tk* and *mtII* (Fig. 2) and the human ribosomal protein S17 (9) genes do not contain DMIs. Second, dense methylation in the two DMIs examined is dynamically regulated with the cells' DNA replication program. In exponentially growing cells actively engaged in chromosomal DNA replication, the DMIs are fully methylated. In nonreplicating $G_0$ cells, methylations at CpA, CpC, and CpT dinucleotides are lost within 48 h and restored rapidly when cell growth is restored.

The two DMIs described in this report share no obvious similarities with respect to their base sequences and compositions except for their surprisingly high contents of 5-methylcytosine. Indeed, $DMI_{dhfr}$ (516 bp) is nearly four times longer than $DMI_{S14}$ (127 bp). This suggests that dense methylation per se is likely to be functionally, as well as structurally, significant. However, it is interesting to note that both DMIs contain three to four times as many CpA and CpT dinucleotides as CpG and CpC dinucleotides (37 versus 9 for $DMI_{S14}$ and 164 versus 51 for $DMI_{dhfr}$). The locations of the DMIs within *ori*-β and $ori_{S14}$ and their relationships to the origins' replication initiation zones and transcribed (i.e., exon) sequences are illustrated in Fig. 7. Interestingly, in both chromosomal origins, the DMIs are located near one end of the replication initiation zones, as determined by biochemical experimental approaches (40).

Although we do not know the DMI's function within a chromosomal replication origin, three possibilities are attractive to consider. First, adenine methylation is known to be important for the activities of prokaryotic chromosomal and episomal replication origins (26), presumably because DNA within the origins bind to plasma membrane only when hemimethylated (2, 22, 29). By analogy, DMIs may mediate analogous associations between mammalian replication origins and the nuclear envelope or scaffolding, despite the fact that they

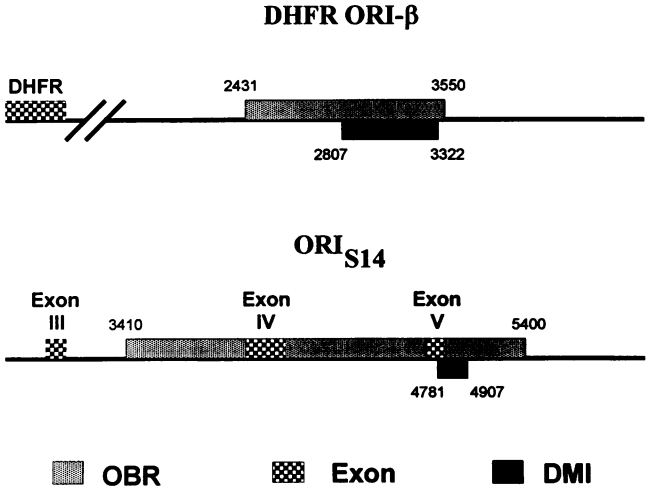


FIG. 7. DMIs within *ori*-β and $ori_{S14}$. Black rectangles, DMIs; checkered rectangles, exons; stippled rectangles, replication initiation zones for *ori*-β (GenBank entry X52034) and $ori_{S14}$ (40). Locations within CHO *ori*-β and $ori_{S14}$ chromosomal DNAs are indexed as in GenBank entries X52034 and M35008, respectively.

contain methylcytosine rather than methyladenine. Indeed, it is known that mammalian replication origins interact with the nuclear envelope (4) and/or scaffolding (11) and that mutational deficiencies in DNA cytosine methylation result in abnormal chromosome replication products in *N. crassa* (12).

Second, metazoan chromosomes contain many more replication origins than are used by a single cell during a particular mitotic cycle. The activities of specific replication origins are regulated, at least in part, by the cell's developmental program and appear to correlate with the transcriptional activities of nearby chromosomal loci. This phenomenon has been termed origin licensing (4). It is possible that dense methylation of DMIs is involved in the origin licensing mechanism. Since the two replication origins in which we have detected DMIs are both programmed to replicate during the first 5 min of S phase at 37°C (6, 15, 40), the DMIs that we describe might reflect an early S license.

Third, during a single S phase, each active chromosomal origin must initiate DNA replication only once in order to ensure binary replication of the entire cellular genome. Redundant initiations at the same chromosomal origin likely would result in the aberrant amplification of surrounding DNA sequences (25, 38). Conversion of bilaterally methylated DMIs to their hemimethylated replication products might be used by the replication machinery to mark previously initiated origins and thereby ensure stoichiometric replication of the entire genome. This model suggests the presence of nuclear factors, probably proteins, that distinguish between fully methylated and hemimethylated DMIs. A few mammalian nuclear proteins which might accomplish this function already have been described (19, 23, 24).

As explained in the accompanying report (40), the CHO-K1 $ori_{S14}$ locus provides an excellent experimental system in which to test these speculations. Because $ori_{S14}$ is contained within a hemizygous region of CHO chromosome 2q (46), as a single-copy locus it is particularly amenable to experimental analyses based on molecular and genetic approaches.

Because of 5-methylcytidine's documented effects on transcription, parental imprinting, and mutagenesis, most previous studies of mammalian DNA methylation have focused on methylation at CpG dinucleotides (reviewed in reference 3). Although CpG is the most abundantly methylated dinucleotide in mammalian DNA, methylcytidine in CpA, CpT, and CpC dinucleotides also has been documented in a variety of eukaryotic DNAs (13, 35, 41, 45). Woodcock et al. quantitatively analyzed the distribution of 5-methylcytidine in human spleen DNA and determined that only 45.5% of the 5-methyl dC in human DNA was contained in CpG dinucleotides (45). CpA, CpT, and CpC dinucleotides accounted for approximately 54.5% of the human genome's cytosine methylation. Unfortunately, little or nothing is known about the functional and/or regulatory significance of non-CpG DNA methylation in higher eukaryotes. From the results of Woodcock et al., it is possible to calculate that human DNA (diploid genome size $\cong 6 \times 10^9$ bp) includes $\sim 2 \times 10^7$ residues of 5-methylcytidine in non-CpG dinucleotides. Therefore, the measured amount of non-CpG methylation in the genome is sufficient to account for approximately $8.5 \times 10^4$ duplex DMI structures with a mean length of 500 bp. Although it is premature to generalize mammalian DMI structures from the two examples described in this report, we note that the result of the calculation presented above is considerably larger than the number of active replication origins in mammalian chromosomes estimated by DNA fiber autoradiography (16) and therefore is more than sufficient to account for DMIs within each active replication initiation zone.

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